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Tentoxin has at least two binding sites on CF_1 and ε-depleted CF_1 ATPases isolated from spinach chloroplast

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Abstract **A new procedure for synthesis of ^{14}C-labeled tentoxin [^{14}C-MePhe[(Z)Δ]^3-tentoxin], with a high specific activity, is described. Binding experiments with CF_1 or CF_1-ε isolated from spinach chloroplast have been carried out using equilibrium dialysis technique. The results show the presence of two classes of binding sites. The association constants of the two major binding sites were derived from non-linear fitting of the binding curves. At 4°C, the first binding site has a value of $K_{a1} = 8.2 \times 10^5$ M^{-1} in CF_1 and 8.7×10^5 M^{-1} in CF_1-ε, while the second binding site has lower affinity with $K_{a2} = 1.5 \times 10^4$ M^{-1} in CF_1 and 2.3×10^3 M^{-1} in CF_1-ε.**

Key words: H$^+$-ATPase; Chloroplast ATP synthase; ^{14}C-Labelled tentoxin; Cyclic tetrapeptide; Equilibrium dialysis; *Spinacia oleracea* L.

1. Introduction

Tentoxin is a natural cyclic tetrapeptide [*cyclo*-(L-MeAla^1-L-Leu^2-MePhe[(Z)Δ]^3-Gly^4)] which is the secondary metabolite of the phytopathogenic fungus *Alternaria alternata*, known to cause chlorosis in many sensitive plants [1]. Tentoxin-induced chlorosis has been observed in seedlings and, in most cases, was proved to result from the inhibition of photophosphorylation by the toxin [2] (for a review see [3]). An identified species-specific receptor for tentoxin is the chloroplast F_0-F_1 H$^+$-ATPase. This enzyme couples the downhill proton flow generated by the photosynthetic redox chain with ATP synthesis. Numerous studies have shown that tentoxin tightly binds to the soluble F_1 moiety of the enzyme [2,4], but very little is known about the mode of action of the toxin. The extrinsic part of chloroplastic ATP synthase, CF_1, is a multi-subunit enzyme (stoichiometry $\alpha_3\beta_3\gamma\delta\varepsilon$) with latent ATPase activity. Removal of the ε-subunit and/or chemical reduction of the lone disulfide bond of the enzyme located on the γ-subunit activates ATPase activity [5]. For sensitive plants, tentoxin binding inhibits the CF_1-ATPase activity at low concentration (10^{-8} M) [2,4,6,7] and stimulates it at a concentration of 10^{-5} M and higher [6–9]. The activation phenomenon also exists in the membrane-bound CF_0-F_1, although it leads to only partial recovery of the ATPase activity [10]. Interestingly, a tightly coupled proton transport is preserved in the partially reactivated CF_0-F_1 by high concentrations of tentoxin [10], and the interaction between the nucleotide binding sites, which plays a important role in the functioning of this complex, is severely altered [11,12].

The precise binding site for tentoxin on CF_1 has not been yet identified. There is some evidence that tentoxin binds to the β-subunit [3,11] although amino acids involved in the binding are still a matter of controversy [5,13]. A binding site located on the α-subunit or at an interface between the subunits α and β is also a possibility [7,14]. In addition, the binding of the γ-subunit to the αβ-complex is necessary for tight binding of tentoxin and for high-affinity, asymmetric nucleotide binding [15].

Using ultrafiltration techniques with a ^{3}H-labeled tentoxin, Steele et al. [2] found a 1:1 stoichiometry for tentoxin-CF_1 binding. But only high affinity binding sites can be detected by ultrafiltration. The possible existence of a second binding site of lower affinity has been suggested [8,9] and we found some evidence for this site using HPLC techniques [7]. The capability of the phytotoxin to inhibit ATPase activity at very low concentrations (< 0.1 μM), as well as to activate it at high concentrations (> 5 μM), prompted us to relate these data to different classes of binding sites. Using equilibrium dialysis with ^{14}C-labeled tentoxin [^{14}C-MePhe[(Z)Δ]^3-tentoxin] synthesized by new methods, we present here an investigation of tentoxin binding on spinach CF_1 and CF_1-ε, which reveals at least two types of binding sites with different affinities.

2. Materials and methods

2.1. ^{14}C-Labelled tentoxin

BocMeAla-Leu-Δ^{Z}Phe-GlyOMe (**1**) was obtained in homogeneous phase using the synthesis scheme recently described [16] skipping the methylation step of the dehydrophenylalanyl residue (Δ^{Z}Phe). HPLC Rt = 23.2 min on C_{18} Protein&Peptide VYDAC analytical column (MeOH/H_2O 60/40 v/v, 0.6 ml min^{-1}). Using [^{14}C]methyl iodide freshly synthesized [17] from [^{14}C]methanol produced from $^{14}CO_2$ (specific activity 55 mCi/mmol) [18,19], selective radioactive methylation was then performed according to [20] affording BocMeAla-Leu-^{14}CMeΔ^{Z}Phe-GlyOMe (**2**) with 80% yield. Purification: Silicagel Merck Si 40-63, ethyl acetate/acetone/hexane 2/1/1, Rf = 0.68, ^{1}H NMR ($CDCl_3$) δ (ppm): 8.54 (1 H, t J = 6 Hz, NH Gly), 7.72 (1H, s, H_β ΔPhe), 7.40 (5H, s, H arom. ΔPhe), 4.67 (1H, large s, NH Leu), 4.31 (1H, dd J = 7.2 and 4.8 Hz, H_α Ala), 4.13 (2H, dd J = 6.0 and 4.13 Hz, CH_2 Gly), 3.71 (3H, s, OCH3), 3.20 (4H, s, N$^{14}CH_3$ ΔPhe H_α Leu), 2.78, 2.69 and 2.56 (3H, 3s, NCH_3 Ala 3 conformations 8, 82 and 10%), 1.65 (2H, s, CH_2 Leu), 1.46 (9H, s, Boc), 1.25 (4H, d and m J = 7.2 Hz, CH_3 Ala and Hγ Leu), 0.57 and 0.55 (2×3H, 2×d J = 4.5 Hz, 2×CH_3 Leu). Radiochemical purity was estimated by thin layer chromatography (ethyl acetate/acetone/hexane 2/1/1, Rf = 0.68)

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Abbreviations: CF_0-F_1, chloroplast ATP synthase (H$^+$-ATPase); CF_1, catalytic sector of the chloroplast H$^+$-ATPase; CF_1-ε, ε-subunit depleted CF_1; Rt, retention time; s, singlet; d, doublet; t, triplet; m, multiplet; eq., equivalent; K_a, association constant; *Enzyme*: ATP synthase (EC 3.6.1.3.)

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(Merck, 0.25 cm, Silicagel 60 F-254): >99%. Mass spectrometry (Finnigan 4600) (CH_4/DCI) confirmed the molecular weight and estimated specific activity to 52 mCi/mmol.

After C- and N-terminal deprotection of (**2**), the cyclization reaction was performed with diphenylphosphoryl azide (DPPA) as described [16]. After purification on Silicagel Merck Si 40-63, ethyl acetate/methanol 9/1, Rf = 0.34, we obtained ^{14}C-MeΔ^{z}Phe^3-tentoxin (**3**) with 19% yield. HPLC (C_{18} Zorbax SB analytical) CH_3OH/H_2O 6/4, 1.0 ml min^{-1}, Rt = 13.3 min. Radiochemical purity (HPLC and TLC): >99%. Specific activity according to mass spectrometry: 52 mCi/mmol.

2.2. Enzyme preparation and equilibrium dialysis

CF_1 was isolated from spinach (*Spinacia oleracea* L.) following standard procedures [21]. The activated complex (CF_1-ε) was produced by removing the ε-subunit by extensive washing of CF_1 bound onto an HPLC anion exchanging column [22]. CF_1 and CF_1-ε were stored at 5°C in 2.6 M ammonium sulfate at a concentration of 15 mg ml^{-1}. The ammonium sulfate precipitate was resolubilized by dialysis against Tris-HCl 20 mM, pH 8.0, $MgCl_2$ 1 mM buffer. Protein concentration was determined using the Bio-Rad protein assay [23] with bovine serum albumin as standard.

For equilibrium dialysis, samples with 3–4 mg ml^{-1} of enzyme (CF_1 or CF_1-ε) were dialysed in SPECTRA/POR tubing (cutoff 12–14 kDa) for 18 h at 4°C to prevent enzyme degradation, against Tris-HCl 20 mM, pH 8.0, $MgCl_2$ 1 mM buffer containing various concentrations of freshly synthesized ^{14}C-tentoxin (ranging from 0.1 μM to 0.1 mM). Control dialysis tubing without enzyme was used to prove that the ^{14}C-tentoxin concentration was the same on both sides of the membrane under these dialysis conditions. The outside and inside radioactivity was measured with a scintillation counter (Wallac 1409). 100 μl of the radioactive solution was mixed in a counting vial with 5 ml of scintillating liquid (Aqualyte from Baker). For each concentration determination, two vials were prepared. Counting was performed during 6 min with ^{14}C cpm, with tentoxin concentration ranging from 1000 ± 30 to $10^6 \pm 3 \times 10^3$. Free tentoxin concentration was deduced from the radioactivity measured outside the dialysis tubing, and bound tentoxin concentration from the difference between the radioactivity measured outside and inside the dialysis tubing. For the determination of the [Bound tentoxin]/[Total enzyme] ratio, final protein concentration inside the dialysis tubing was measured in duplicate. To verify the enzyme stability during the experience, the ATPase activity of CF_1-ε was checked as described [24] and was comparable before and after the equilibrium dialysis without tentoxin (5.0 μmol ATP min^{-1} mg^{-1} CF_1-ε).

Binding curve fitting was performed using ORIGIN 3.5 software which uses Levenberg-Marquardt algorithm and simplex method [25]. Different models including a maximum of two ligands and two classes of sites were tested.

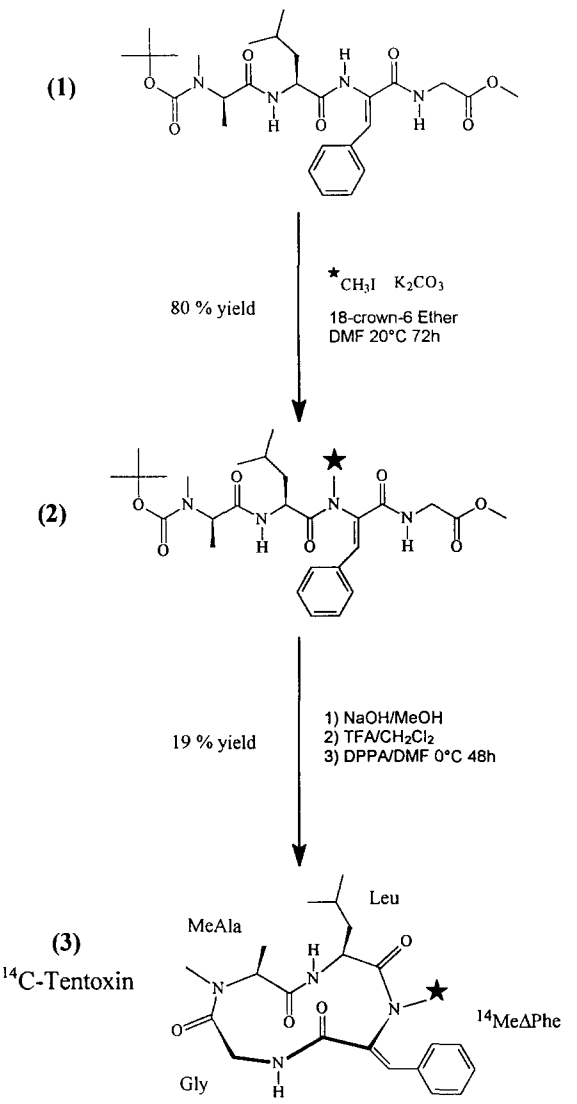

Fig. 1. Synthesis of N^{14}CH$_3$Δ^{z}Phe^3-tentoxin. The star identifies the ^{14}C position.

3. Results and discussion

A radiolabeled ligand of sufficient radiochemical specific activity, purity and stability, is essential to any equilibrium dialysis study of ligand-receptor binding. Although the biosynthesis of radiolabeled tentoxin is possible [26], the accessible specific activity is generally too low for a reliable determination of tentoxin binding to CF_1 [7]. The total organic synthesis of tentoxin, which is well-documented [16,20,26–28], allowed us to produce highly radioactive cyclic tetrapeptide in sufficient amounts. In order to obtain high specific activity and to avoid the problems due to exchange of ^{3}H labels, we chose to prepare ^{14}C-labeled tentoxin using the synthetic pathway described in Fig. 1 where tentoxin is specifically methylated with $^{14}CH_3I$ on the nitrogen atom of the dehydrophenylalanine residue.

By comparison to other methods like ultrafiltration [2] and HPLC [7], which are only suitable for rapidly achieved equilibria, equilibrium dialysis is a method of choice for the study of slowly saturated low-affinity binding sites (incubation time >45 min; $K_a < 10^6$ M^{-1}). The equilibrium dialysis measurement does not disturb the equilibrium between the free and the bound ligand. In the case of tentoxin, the ultrafiltration and HPLC techniques lead some to conclude a 1:1 binding of the toxin on the CF_1. The HPLC technique that we used, the Hummel and Dreyer method, suggested the presence of a second low-affinity binding site (with an estimated $K_a = 6.3 \times 10^3$ M^{-1}). In order to establish the existence of a second site, equilibrium dialysis experiments were performed with radioactive tentoxin binding to either CF_1 or CF_1-ε.

Fig. 2 shows the binding of tentoxin to CF_1. At high tentoxin concentrations, two toxin molecules per CF_1 are bound. The presence of two different classes of binding sites was tested using a non-linear fitting of the binding data (see Fig. 2). First, we considered a model which involved two sites having the same binding constant (two-site one-K_a model). The non-linear best-fit of the experimental data using this model yielded an unsatisfactory fit (dashed line, chi-square fitting $\chi^2 = 8.2 \times 10^{-2}$). Next, we allowed the number of sites to vary, keeping the binding constants the same for all sites

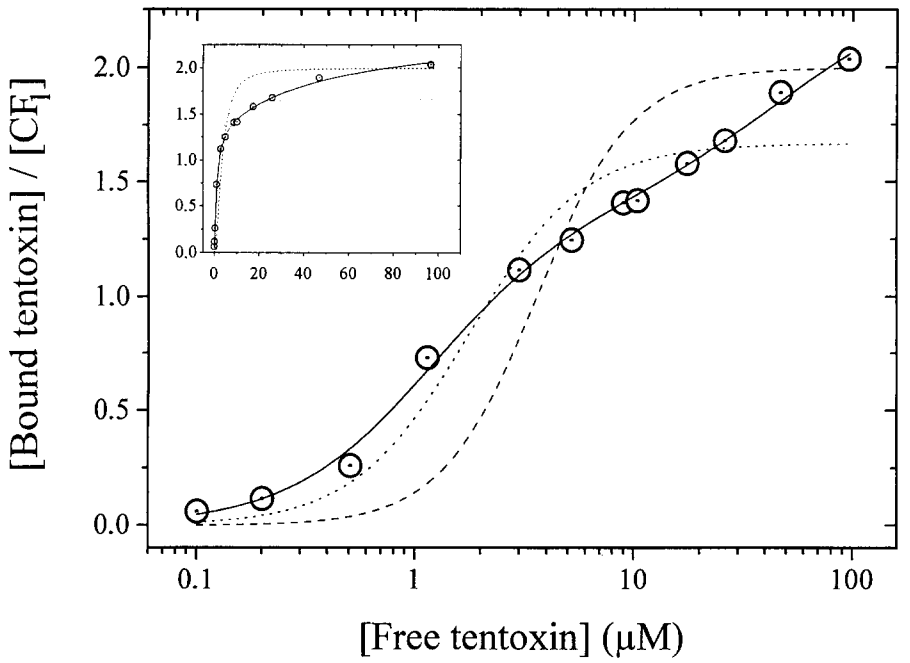

Fig. 2. Non-linear fitting of equilibrium dialysis data for ^{14}C-tentoxin binding to spinach CF_1. In the inset are represented the same fits, with linear abcissa scale. (⊙), experimental points. Plain line: best fit obtained using a *n*-site two-K_a model ($\chi^2 = 1.5 \times 10^{-3}$, 1.3 ± 0.05 sites with $K_{a1} = 8.2 \pm 0.9 \times 10^5$ M^{-1} and 1.1 ± 0.1 sites with $K_{a2} = 1.5 \pm 0.8 \times 10^4$ M^{-1}). Dotted line: best fit obtained using a *n*-site one-K_a model ($\chi^2 = 3.3 \times 10^{-2}$, 1.7 ± 0.07 sites with $K_a = 3.9 \pm 1.5 \times 10^5$ M^{-1}). Dashed line: best fit obtained using a two-site one-K_a model ($\chi^2 = 8.2 \times 10^{-2}$, $K_a = 7.7 \pm 3.2 \times 10^4$ M^{-1}).

(*n*-site one-K_a model). A better fit (dotted line, $\chi^2 = 3.3 \times 10^{-2}$) was obtained when the number of sites was 1.7. However, the inflection of the curve was still not well-described by this model, neither were the high concentration data points. Finally, we considered a model which consisted of two classes of sites. Within each class, the number of sites and the association constants were allowed to vary (*n*-site two-K_a model). The best fit of the experimental data yielded: 1.3 ± 0.05 bind-

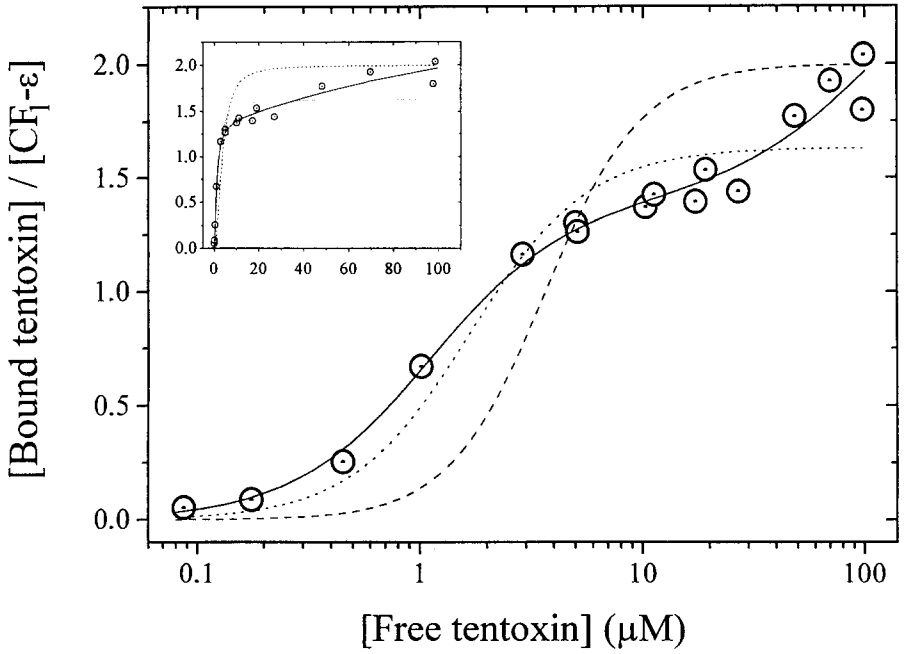

Fig. 3. Non-linear fitting of equilibrium dialysis data for ^{14}C-tentoxin binding to spinach CF_1-ε. In the inset are represented the same fits, with linear abcissa scale. (⊙), experimental points. Plain line: best fit obtained using a *n*-site two-K_a model ($\chi^2 = 6.2 \times 10^{-3}$, 1.4 ± 0.06 sites with $K_{a1} = 8.7 \pm 1.7 \times 10^5$ M^{-1} and 1.3 ± 0.3 sites with $K_{a2} = 2.3 \pm 3.8 \times 10^3$ M^{-1}). Dotted line: best fit obtained using a *n*-site one-K_a model ($\chi^2 = 3.6 \times 10^{-2}$, 1.6 ± 0.06 sites with $K_a = 4.3 \pm 1.6 \times 10^5$ M^{-1}). Dashed line: best fit obtained using a two-site one-K_a model ($\chi^2 = 1.1 \times 10^{-1}$, $K_a = 7.5 \pm 2.9 \times 10^4$ M^{-1}).

ing sites with a $K_{a1} = 8.2 \pm 0.9 \times 10^5$ M^{-1} and 1.1 ± 0.1 binding sites with a lower affinity of $K_{a2} = 1.5 \pm 0.8 \times 10^4$ M^{-1} ($\chi^2 = 1.5 \times 10^{-3}$, plain line).

For the activated enzyme with the ε-subunit removed (CF_1-ε), similar results were found as shown in Fig. 3. The fit using the two-site one-K_a model was poor ($\chi^2 = 0.1$). The *n*-site one-K_a model did not describe the high toxin concentrations satisfactorily (1.6 binding sites, $\chi^2 = 3.6 \times 10^{-2}$). Again, the fit using the *n*-site two-K_a model yielded the best fit of the experimental data ($\chi^2 = 6.2 \times 10^{-3}$): 1.4 ± 0.06 binding sites with a $K_{a1} = 8.7 \pm 1.7 \times 10^5$ M^{-1} and 1.3 ± 0.3 sites with a lower affinity of $K_{a2} = 2.3 \pm 3.8 \times 10^3$ M^{-1}.

From these data we concluded that:

(i) two classes of toxin binding sites are present on both enzymes,

(ii) very similar affinity constants of the first site (K_{a1}) are found for CF_1 and CF_1-ε,

(iii) the binding constants K_{a2} of the second site appears to be different. However, the larger uncertainty in the estimation of K_{a2} for the CF_1-ε case could account for this. The experimental data were indeed more scattered for high toxin concentrations and, hence, the error in the determination of K_{a2} was higher.

From this analysis, we can account for at least two tentoxin binding sites in spinach CF_1. Another result is that two tentoxin binding sites are found in CF_1 either in the presence or in the absence of the ε-subunit. This subunit seems not essential for the binding of tentoxin. However, its absence might weaken the affinity for the second site. We checked that the enzyme is stable with time at 4°C, by measuring the ATPase activity before and after the 18 h dialysis experiment. The ATPase activity did not evolve within this period of time, indicating that the slight difference observed in the K_{a2} values was not due to some denaturation in the enzyme structure.

The presence of two types of binding sites can be related to the known tentoxin properties of inhibition of the CF_1 ATPase activity below 0.1 μM and of reactivation above 5–10 μM. The calculated association constant for the second site can be correlated to the concentration range for reactivation of the ATPase activity. However, the high-affinity binding constant K_{a1} seems to be one order of magnitude too low to explain the ATPase inhibition below 0.1 μM. Nevertheless, since the experiments were carried out at low temperature (4°C) and not at 37°C where enzymatic ATPase activity is usually measured, this result is not unexpected.

Hence, in our model, we relate the first binding site to the inhibition effect, and the second binding site to the stimulation process observed at higher tentoxin concentration.

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